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REVIEW

Targeting tRNA methyltransferases: from molecular mechanisms to drug discovery

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Transfer RNA methyltransferases (tRNA MTases) catalyze site-specific methylation on tRNAs, a critical process that ensures the stability and functionality of tRNA molecules, thereby maintaining cellular homeostasis of tRNA methylation. Recent studies have illuminated the structural diversity, specific substrate recognition, and conserved catalytic mechanisms of tRNA MTases, revealing how their dysregulation contributes to various diseases, including cancers and neurodevelopmental disorders. This review integrates these advances, exploring the challenges of achieving precise substrate recognition and modification in the context of complex and specific tRNA modification landscape, while emphasizing the crucial role of tRNA MTases in disease pathogenesis. The identification of small-molecule inhibitors targeting specific tRNA MTases marks a promising step toward the development of novel therapies. With continued research into the broader biological functions and regulatory mechanisms of tRNA MTases, these insights hold great potential to drive clinical advancements and therapeutic innovations.

tRNA methylation | tRNA methyltransferase | tRNA recognition | catalytic mechanism | drug development

Introduction

tRNA is extensively modified by various chemical modifications that are crucial for its stability, maturation, and function (Phizicky and Hopper, 2023; Suzuki, 2021). Among these modifications, methylation is one of the most prevalent, distributed throughout the tRNA molecule (Cappannini et al., 2024; Lei et al., 2023). tRNA methyltransferases (MTases) are the enzymes responsible for transferring methyl groups from the S-adenosylmethionine (SAM) methyl donor to specific tRNA nucleotides (Hou and Perona, 2010). To modify distinct tRNAs at precise locations, tRNA MTases have evolved diverse structural features and substrate recognition mechanisms (Swinehart and Jackman, 2015). Dysregulation of tRNA MTases can result in aberrant methylation patterns, affecting tRNA stability and translation efficiency, and has been associated with a range of diseases (Delaunay et al., 2024). As the understanding of tRNA MTases deepens, new insights into their role in disease pathogenesis continue to emerge, highlighting the therapeutic potential of targeting these enzymes. This has opened new avenues for disease treatment and drug discovery. In this review, we will explore recent progress in understanding the molecular mechanisms of tRNA MTases, their involvement in disease, and their potential as therapeutic targets.

Highly regulated tRNA methylations in human

To date, over 100 distinct modifications have been identified in human tRNAs, with the average tRNA containing approximately 13 modified nucleotides (Cappannini et al., 2024; Lei et al., 2023; Pan, 2018; Smith et al., 2024). Among these modifications, methylation is the most prevalent and is conserved across a wide range of organisms, playing a critical role in maintaining tRNA stability and function. Methylation occurs at various positions throughout the tRNA molecule, including the acceptor arm, D-arm, T-arm, anticodon arm, and variable loop (Phizicky and Hopper, 2023; Suzuki, 2021). According to the RNA modification databases, about 22 types of methylations have been annotated in human cytoplasmic tRNAs and eight types in mitochondria tRNAs (Figure 1A–C) (Boccaletto et al., 2022; Lei et al., 2023).

Primary tRNA transcripts undergo a series of precisely regulated post-transcriptional processing steps (Hopper, 2013; Johansson and Byström, 2002). Methylation is one of the key post-transcriptional modifications in tRNA and occurs through a highly coordinated process that ensures the proper maturation of tRNA molecules. Certain tRNA methylations, like m^7G46, are generated immediately after transcription (Tomikawa, 2018). Some others occur in a sequential and highly coordinated manner, involving intricate cross-talk between different matura-

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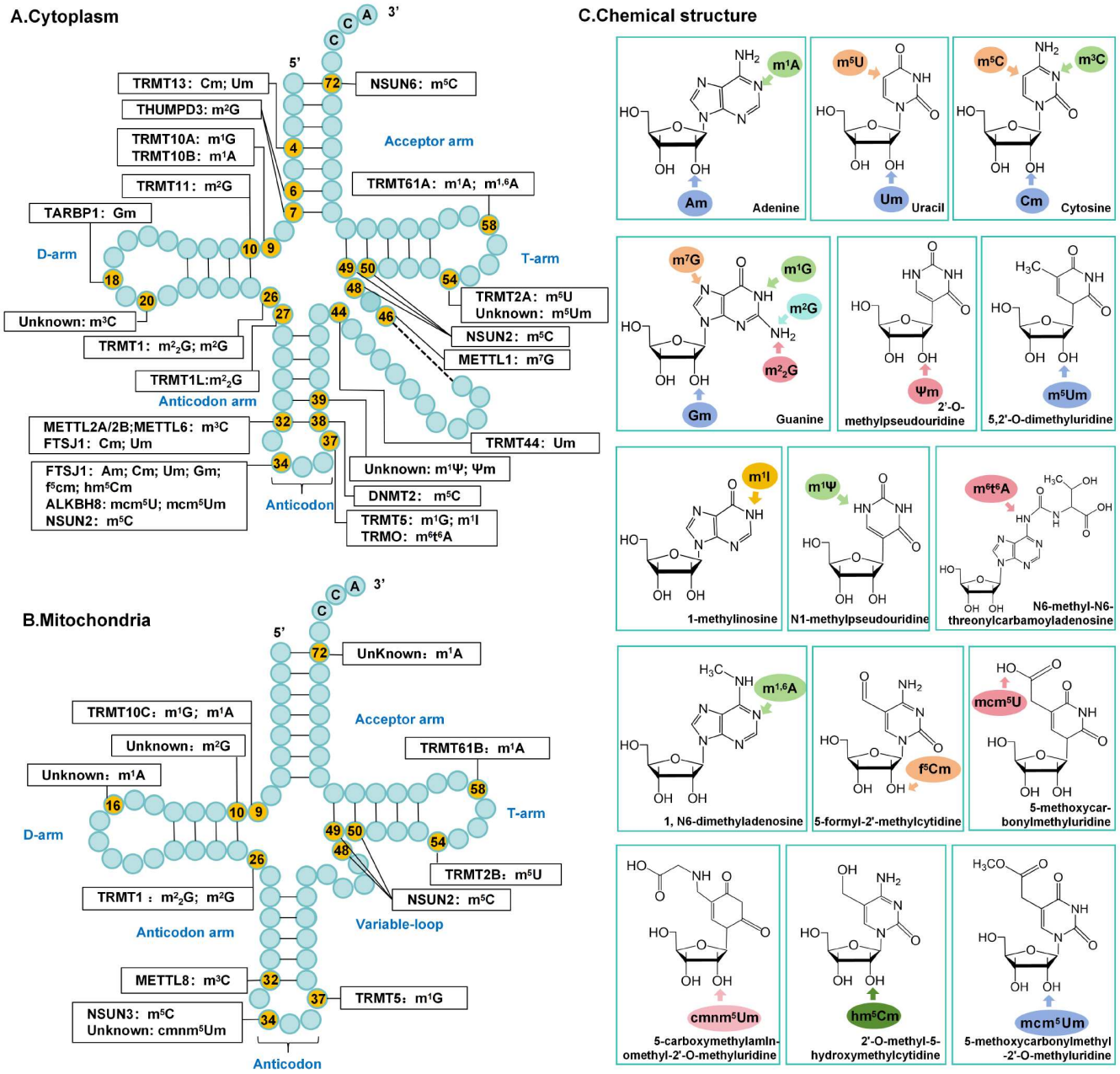

Figure 1. Map of human tRNA methylations. A and B, Methylations of human cytoplasmic and mitochondrial transfer RNAs (tRNAs). Nucleotides under methylations are colored yellow, and the relevant enzymes are indicated (Suzuki, 2021). C, Locations of methyl groups transferred onto nucleotide for each methylation type. All these modifications are described in MODOMICS (https://genesilico.pl/modomics/) and tmodbase (https://www.tmodbase.com/#/) databases. m^1A, 1-methyladenosine; Am, 2′-O-methyladenosine; m^5U, 5-methyluridine; Um, 2′-O-methyluridine; m^3C, 3-methylcytidine; m^5C, 5-methylcytidine; Cm, 2′-O-methylcytidine; m^1G, 1-methylguanosine; m^2G, N2-methylguanosine; $m^2{}_2G$, N^2,N^2-dimethylguanosine; m^7G, 7-methylguanosine; Gm, 2′-O-methylguanosine; Ψm, 2′-O-methylpseudouridine; m^5Um, 5,2′-O-dimethyluridine; m^1I, 1-methylinosine; $m^1Ψ$, 1-methylpseudouridine; m^6t^6A, N^6-methyl-N^6-threonylcarbamoyladenosine; $m^{1.6}A$, 1,N^6-dimethyladenosine; f^5Cm, 5-formyl-2′-O-methylcytidine; mcm^5U, 5-methoxycarbonylmethyluridine; $cmnm^5Um$, 5-carboxymethylaminomethyl-2′-O-methyluridine; hm^5Cm, 2′-O-methyl-5-hydroxymethylcytidine; mcm^5Um, 5-methoxycarbonylmethyl-2′-O-methyluridine.

tion and modification events (Li et al., 2021a). For example, the formation of 5-methylcytosine at position 38 requires the modification of inosine at the wobble position (I34) in $tRNA^{Val}$ (Huang et al., 2021); the modification of t^6A37 is a prerequisite for m^3C32 deposition in $tRNA^{Thr}$ (Mao et al., 2021); the m^5C modification at the wobble position 34 of mitochondrial $tRNA^{Met}$, which is further required for f^5C34 biogenesis (Nakano et al., 2016).

tRNA methylations generally contribute to tRNA stability and function, with some modifications playing a crucial role in maintaining structural integrity and proper tRNA function. However, the extent of their impact varies, as certain modifications are essential for specific tRNA functions, while others have more subtle regulatory roles. Notably, demethylation processes have also been identified in tRNA, highlighting the reversible nature of these modifications (Chen et al., 2019; Kawarada et al.,

2017; Ueda et al., 2017; Wei et al., 2018; Zhang et al., 2021a). Thus, tRNA methylations are dynamically regulated in response to cellular needs, environmental stress, and disease states (Gao et al., 2022; Hughes et al., 2024; Phizicky and Hopper, 2023; Suzuki, 2021). The complexity and specificity of tRNA methylation in humans reflect the highly regulated nature of this modification process. Diverse methyltransferases, precise site-specific modifications, and regulatory mechanisms ensure that tRNA methylation supports the structural integrity, stability, and functionality of tRNA molecules.

Functions of tRNA methylation

tRNA methylations serve two primary functions: they stabilize the tertiary structure of tRNA, promoting proper folding and interactions with processing, modification, and aminoacylation enzymes, as well as tRNA surveillance machinery. Additionally, they enhance the accuracy of codon-anticodon recognition, thereby improving the efficiency of protein synthesis (Agris et al., 2007; Tuorto and Lyko, 2016; Väre et al., 2017). These modifications are primarily distributed in two regions: the core, which includes the junction of the T-arm, D-arm, and variable loop (VL), and the anticodon loop (ACL) region of the tRNA (Zhang and Lu, 2025).

Modifications in the tRNA core tend to be simpler and primarily stabilize the L-shaped structure by reinforcing interactions between the T-arm, D-arm, and variable loop, fine-tuning the overall stability of the molecule (Berg and Brandl, 2021). In humans, most cytoplasmic tRNAs containing G26 are modified with $m^2{}_2G26$. This modification allows G26 to form non-Watson-Crick base pairs with A/U44, acting as a molecular hinge that promotes proper tRNA folding and enhances stability (Figure 2A, left) (Bavi et al., 2011; Urbonavičius et al., 2006). The m^1A58 modification is critical for stabilizing the tertiary structure of tRNAs, particularly in eukaryotic initiator tRNAs, where it creates a substructure by forming hydrogen bonds with adenosines A20, A54, and A60, contributing significantly to correct tRNA folding (Figure 2A, middle) (Anderson and Droogmans, 2005). Loss of m^1A58 destabilizes tRNA, making it more prone to degradation. Similarly, the m^7G46 modification in the variable loop forms a tertiary base pair with C13-G22, further stabilizing the central tRNA core, and its loss is associated with rapid tRNA degradation (Figure 2A, right) (Alexandrov et al., 2006; Tomikawa, 2018). In mitochondria, the m^1A9 modification prevents the formation of a Watson-Crick A9-U64 base pair, allowing proper folding of the T and D arm, which is critical for the correct folding of mt-$tRNA^{Lys}$ (Figure 2B) (Helm et al., 1998). Additionally, methylations also play important roles in regulating tRNA fragment biogenesis that affects many cellular processes (reviewed elsewhere (Guzzi and Bellodi, 2020; Kuhle et al., 2023; Lyons et al., 2018; Muthukumar et al., 2024)).

Modifications in the anticodon loop region are typically complex and essential for ensuring the rapid and accurate decoding of mRNA sequences by the translation machinery (Hoffer et al., 2020). For example, the m^5C modification at the wobble position 34 of mitochondrial $tRNA^{Met}$, which is further oxidized to f^5C34, broadens the decoding capacity of a single mitochondrial $tRNA^{Met}$ to recognize different methionine codons, facilitating efficient translation within mitochondria (Figure 2C) (Haag et al., 2016). While, in the cytoplasm, the absence of m^5C38 modification may result in incorrect amino acid incorporation, such as glutamate (Glu) being misincorporated in place of aspartate (Asp), leading to translation errors (Figure 2D) (Tuorto et al., 2015). Additionally, deficiency in the m^3C32 modification leads to increased ribosomal occupancy at the A-site for serine codons, which causes translation stalling and significantly reduces mRNA translation efficiency (Figure 2E) (Cui et al., 2024). Furthermore, the m^1G modification at position 37 plays a critical role in preventing ribosomal frameshifting, thereby maintaining the correct mRNA reading frame and ensuring proper polypeptide expression (Figure 2F) (Hoffer et al., 2020).

tRNA methyltransferases in human

Methylation of tRNA is catalyzed by a diverse group of MTases, some of which require cofactors or protein partners to achieve substrate specificity, adding another layer of complexity to the regulation of tRNA methylation. Dysregulation of tRNA MTases can lead to aberrant tRNA methylation patterns, affecting the stability and function of tRNAs (Bavi et al., 2011; Cui et al., 2024). Currently, 28 tRNA MTases have been identified in humans, all utilizing SAM as the methyl group donor. These enzymes belong to the largest family of AdoMet-dependent methyltransferases and are classified into eight classes (class I-VIII) based on their structural folds and evolutionary origins. Among these, 23 tRNA MTases are categorized under class I, characterized by a conserved Rossmann fold in their catalytic domain, though they exhibit diverse mechanisms for recognizing different tRNA substrates. Four MTases belong to class IV, known as the SPOUT family, distinguished by a deep topological knot within the catalytic domain. TRMO, responsible for N6-methyl-N6-threonylcarbamoyladenosine (m^6t^6A) modification, is the sole representative of class VIII (Table 1).

Most human tRNA MTases have been identified; however, only a limited number have been structurally characterized with their tRNA substrates. Given the conserved and rigid L-shaped structure of tRNA, tRNA MTases must employ diverse strategies to recognize and modify specific tRNAs at precise locations within the cellular environment. Despite these differences in substrate recognition, tRNA MTases share common catalytic features. Most utilize an SN2-type nucleophilic substitution reaction, where the tRNA's acceptor nucleotide serves as the nucleophile, and SAM acts as the methyl donor (Hou and Perona, 2010). In this section, we summarize the substrate recognition and catalytic mechanisms of human tRNA MTases based on existing structural data.

TRMT61A/TRMT6: distorted tRNA to deposit the m^1A58

The m^1A modification is one of the most common modifications in tRNAs, with primary modification sites at positions 9 and 58 in human tRNA (Smoczynski et al., 2024). The m^1A58 MTase in humans is composed of two subunits, the catalytic TRMT61A and the non-catalytic TRMT6 (Ozanick et al., 2005). The crystal structure of the TRMT61A/TRMT6 complex bound to a tRNA substrate provides the first insight into how human MTases bind and recognize tRNA (Figure 3A) (Finer-Moore et al., 2015). Notably, the MTase complex must access adenosine 58 (A58), a base buried within the tRNA core region. To achieve this, the human m^1A58 tRNA MTase forms a heterotetramer, enabling the remodeling and catalysis of the tRNA. Upon binding, the

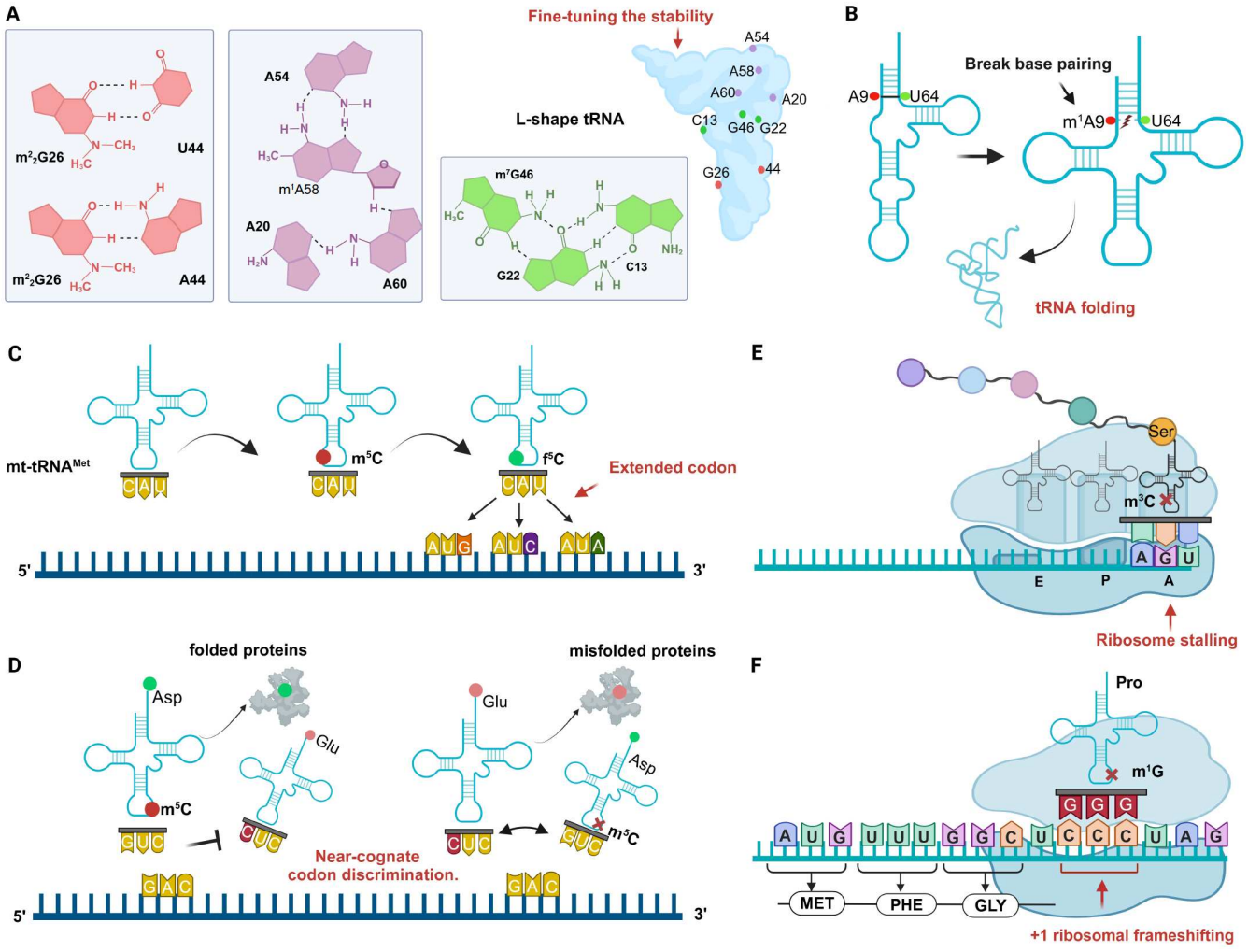

Figure 2. Roles of methylations on tRNA. A, Methylations that stabilize tRNA by base-pairing. The $m^2{}_2G$ modification allows non-Watson-Crick base pairing of G26 with A44 or U44 to enhance stability; m^1A58 forming hydrogen bonds with A20, A54, and A60 to maintain the tertiary structure; m^7G46 stabilizes the L-shaped structure of tRNA by forming a tertiary base pair with the C13-G22 nucleotides. Locations of relative modifications are represented with dots in the L-shape structured tRNA. B, Methylation that ensures the tRNA folding by disrupting base-pairing. m^1A9 modification prevents the unnecessary Watson-Crick base pair (A9-U64) in mt-$tRNA^{Lys}$, ensuring correct folding. C–F, Methylations that ensure rapid and accurate mRNA decoding. The formation of m^5C initiates the biogenesis of 5-formylcytidine (f^5C34), expanding the wobble position of mt-$tRNA^{Met}$ to recognize different codons for methionine (AUG, AUC and AUA) (C). Lack of m^3C38 methylation in $tRNA^{Asp}$ reduces the ability of $tRNA^{Asp}$ to compete with near-cognate codons tRNAs, increasing miscoding by Glu (D). Deficiency of m^3C modification in $tRNA^{Ser}$ results in ribosome stalling at serine codons and decreases mRNA translation (F). The absence of m^1G37 in $tRNA^{Pro}$ leads to a +1 translation frameshifting (H).

D-arm and T-arm of the tRNA are detached from each other by TRMT61A and TRMT6, with the most extensive interactions occurring in the 3′ half of the tRNA (Figure 3A). With this remodeling conformation of tRNA, the A58 is positioned into the catalytic pocket of TRMT61A. Even no direct interactions were observed in crystal structures, Asp181 is close to the N1 atom of A58, suggested as the catalytic residue to assist with the SN2 nucleophilic reaction (Figure 4A) (Finer-Moore et al., 2015).

However, several questions remain unresolved: while the large electropositive surface of the MTase distorts the tRNA structure to facilitate catalysis, the mechanism underlying substrate recognition specificity is not yet fully understood. Moreover, only one A58 base is located in the active pocket of the heterotetramer, leaving the A57 in the second active site (Finer-Moore et al., 2015). The "half-of-the-sites" enzyme mechanism proposed by the authors also requires further investigation. Nevertheless, this study significantly advances our understanding of how internal sites in folded tRNA are modified.

TRMT10C/SDR5C1: accommodating purines for N^1-methylation

The enzymes responsible for m^1A9 modification are TRMT10B and TRMT10C, both members of the SPOUT family (Howell et al., 2019; Vilardo et al., 2020). The TRMT10C/SDR5C1 complex plays a crucial role in mitochondrial tRNA maturation by catalyzing the N^1-methylation of purine at position 9 (m^1A9 or m^1G9) in mitochondrial tRNAs. Structural studies reveal that TRMT10C recognizes most regions of the pre-tRNA, while SDR5C1, although interacting less extensively with the tRNA, serves as a scaffold, anchoring TRMT10C and stabilizing the pre-tRNA (Figure 3B). The target purine at position 9 is flipped into the active site of TRMT10C, where it is stabilized by conserved residues, ensuring the specific selection of purines over pyrimi-

Table 1. Classification of typical S-adenosyl-L-methionine (SAM)-dependent human tRNA methyltransferases

Modification	Position	Methyltransferase		Experimental structure
		Cytoplasm enzyme	Mitochondria	
Class I				
m^1A	58	TRMT61A/TRMT6	TRMT61B	TRMT61A/TRMT6/tRNA: 5CD1
$m^{1,6}A$	58	TRMT61A/TRMT6		
m^3C	32	METTL2A/DALRD3	METTL8/TARS2 METTL8/SARS2	
	32	METTL2B/DALRD3		
	32	METTL6/SerRS		METTL6/SerRS/tRNA: 8P7B
m^5C	34	NSUN2		
	38	DNMT2		
	48/49/50	NSUN2	NSUN2	
	72	NSUN6		NSUN6: 5WWQ. NSUN6/tRNA: 5WWT; 5WWS
	34		NSUN3	
m^1G	37	TRMT5	TRMT5	
	6/7	THUMPD3/TRMT112		
m^2G	10	TRMT11/TRMT112		
$m^2{}_2G$	26	TRMT1	TRMT1	
	26	TRMT1	TRMT1	
	27	TRMT1L		
m^7G	46	METTL1/WDR4		METTL1: 3CKK METTL1/WDR4: 7U20 METTL1/WDR4/tRNA: 8CTI
m^5U	54	TRMT2A	TRMT2B	
Cm	4	TRMT13		
	32	FTSJ1/THADA		
hm^5Cm	34	FTSJ1/WDR6		
f^5Cm	34	FTSJ1/WDR6		
Um	4	TRMT13		
	32	FTSJ1/THADA		
	44	TRMT44		
mcm^5Um	34	ALKBH8/TRMT112		
Nm	34	FTSJ1/WDR6		
	10/34?	FBL		
Class IV				
m^1A	9	TRMT10B	TRMT10C/SDR5C1	TRMT10C/SDR5C1/pre-tRNA: 8CBO; 8CBK
m^1G	9	TRMT10A	TRMT10C/SDR5C1	
Gm	18	TARBP1		
Class VIII				
m^6t^6A	37	TRMO		

dines, contributing to TRMT10C's dual activity (Figure 3B). In the catalytic center, the Asp314 of TRMT10C forms a hydrogen bond with the NH_2 group of A/G9, positioning the substrate for methyl transfer. Structural studies have implicated Asp314 as a potential general base that may facilitate an SN2 nucleophilic attack on the methyl group of the SAM donor, though this remains a hypothesis based on structural inference rather than direct enzymatic evidence (Figure 4B) (Krishnamohan and Jackman, 2017; Meynier et al., 2024; Oerum et al., 2018; Vilardo et al., 2012). Notably, cryo-EM structures reveal an additional TRMT10C-tRNA pair with poor density on the opposite side of the SDR5C1 tetramer, suggesting the possibility that two pre-tRNAs may be processed simultaneously. Furthermore, this complex remains associated with tRNA throughout various processing steps, facilitating coordinated maturation and providing a valuable understanding of how tRNA modifications and sequential maturation occur in mitochondria (Cipullo et al., 2021).

METTL6/SerRS: defining tRNA specificity via seryl-tRNA synthetase

The m^3C modification is predominantly found at position 32 in the anticodon loop of tRNAs and occasionally at position 20 in the D-loop (Cui et al., 2021). In humans, four class I MTases: METTL2A/B, METTL6, and METTL8 are responsible for m^3C methylation (Xu et al., 2017). METTL2A/B modifies cytoplasmic $tRNA^{Thr}$ and $tRNA^{Arg}$ along with DALRD3 (Lentini and Fu, 2019). METTL6 requires seryl-tRNA synthetase (SerRS) to promote m^3C formation in $tRNA^{Ser}$, while METTL8 modifies

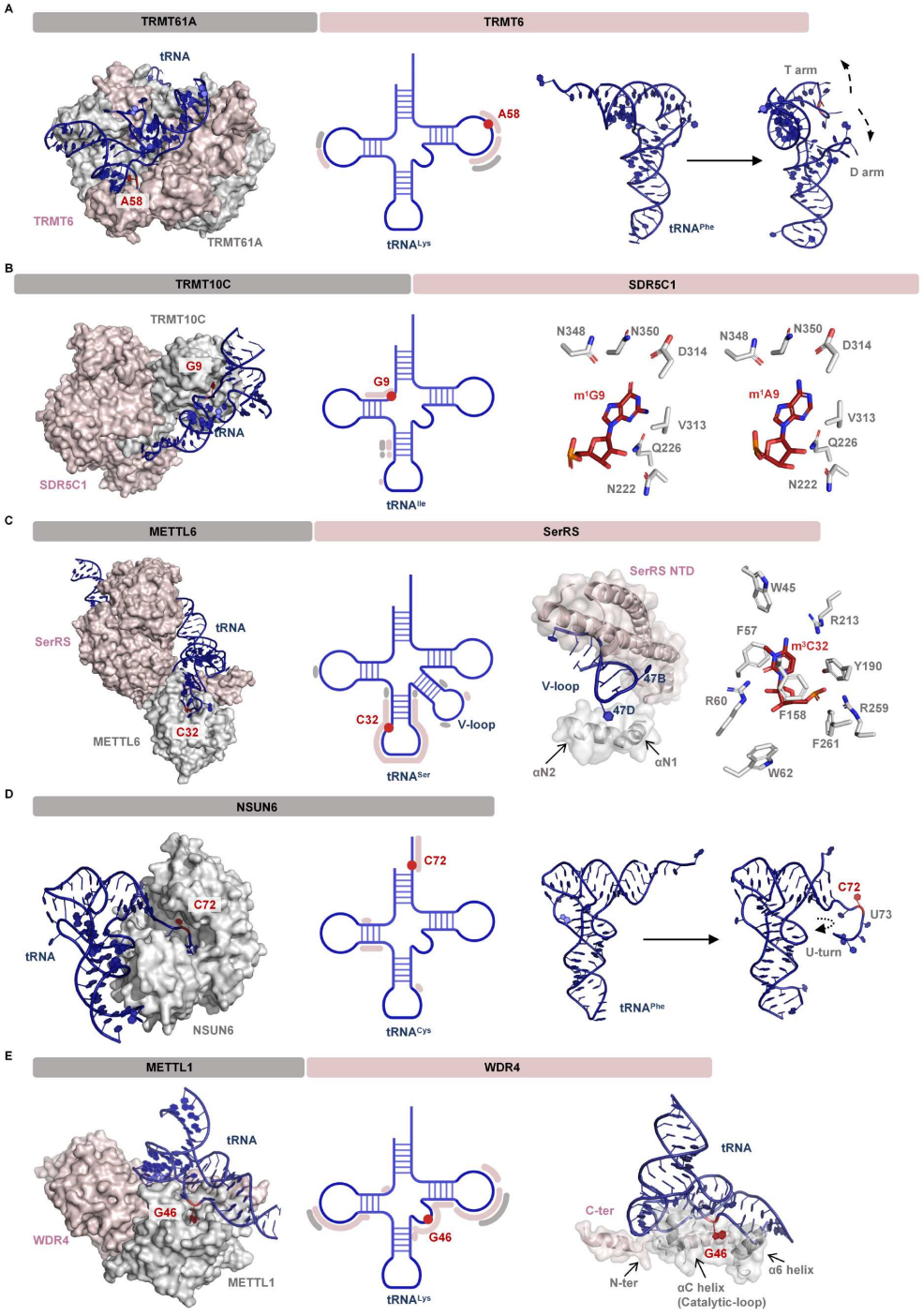

Figure 3. The diverse modes of tRNA MTases in recognizing tRNA. A, TRMT61A/TRMT6 distorts tRNA for m^1A58 modification. In the crystal structure of TRMT61A-TRMT6-$tRNA^{Lys}$ (PDB ID: 5CD1), TRMT61A and TRMT6 primarily recognize the T-arm and D-arm of $tRNA^{Lys}$, causing significant conformational changes to position A58 into the catalytic pocket. The typical tRNA structure $tRNA^{Phe}$ is shown (left, PDB ID: 1EHZ). B, TRMT10C/SDR5C1 recognizes G9 or A9 for methyl group deposition. In the CryoEM structure of TRMT10C-SDR5C1-$tRNA^{Ser}$ (PDB ID: 8CBO), the strong interactions are formed between 5′-half of tRNA and the enzyme. Conserved residues in the catalytic pocket of TRMT10C specifically recognize the G9 or A9 (A9 in pre-$tRNA^{His-Ser}$ are shown, PDB ID: 8CBK). C, METTL6/SerRS modify $tRNA^{Ser}$ for m^3C32 modification. In the CryoEM structure of METTL6-SerRS-$tRNA^{Ser}$ (PDB ID 8P7B), METTL6 and SerRS specifically recognized the distinctive variable loop of tRNA via αN1-αN2 helices and the NTD (N-terminal domain), respectively. METTL6 also binds the anticodon arm of tRNA and deposits the m^3C32 with the conserved residues in the catalytic pocket. D, NSUN6 induces a U-turn tail of tRNA for m^5C72 modification. In the crystal structure of NSUN6-$tRNA^{Cys}$ (PDB ID: 5WWR), NSUN6 recognizes multiple tRNA regions, inducing significant conformational change in 3′ end (Compared with the free form of tRNA on the left, yeast $tRNA^{Phe}$, PDB ID: 4TNA). The U73 also acts as a discriminator for the NSUN6 catalytic process. E, METTL1/WDR4 secures tRNA for m^7G46 modification. In the CryoEM structure of METTL1-WDR4-$tRNA^{Lys}$ (PDB ID:8EG0), METTL1 and WDR4 recognize the tRNA core via multiple elements, including the αC helix (catalytic loop), α6 helix, the N terminal region (N-ter) of METTL1 and the C-terminal region (C-ter) of WDR4. Schematic representations of the catalytic subunit (gray) and non-catalytic subunit (light pink) for each tRNA MTase are illustrated at the top. The overall structures of tRNA MTase bound to their substrate tRNA are displayed on the left. The enzymes and tRNAs are shown in surface and stick representation, respectively. The subunits are colored according to the schematic representation, and the modified nucleotide is highlighted in red. In the middle, secondary structure diagrams of tRNA and their interactions with corresponding enzymes are presented. These interactions are indicated with strips or dots, with colors matching the schematic subunit representations. Modified nucleotides are labeled with red dots.

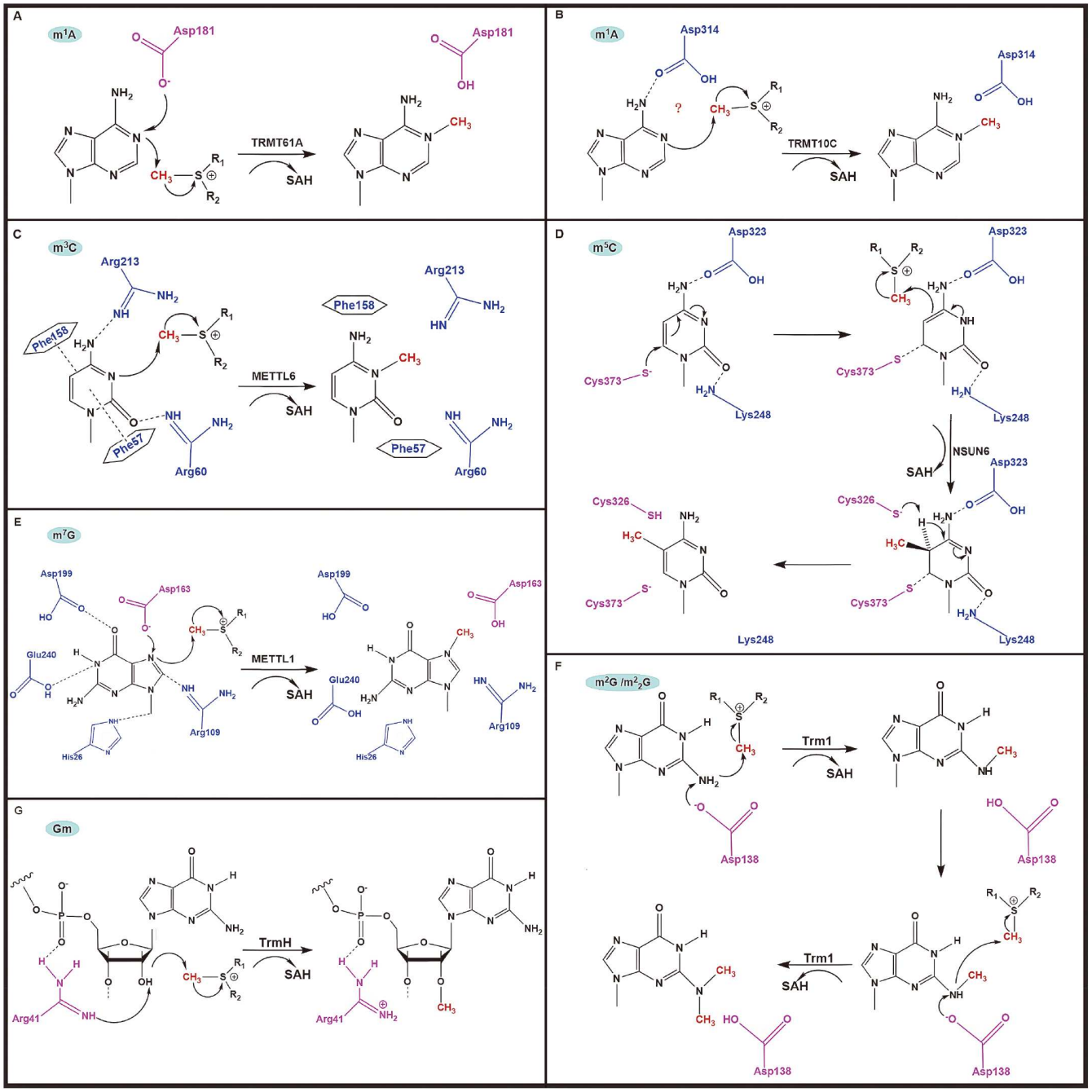

Figure 4. The catalytic mechanisms of tRNA methyltransferase. A–E, Human tRNA MTases exhibit conserved SN2-type nucleophilic reactions. In TRMT61A, Asp181 is positioned near the N1 atom of A58, potentially acting as a catalytic residue to facilitate the SN2 nucleophilic reaction (A). In TRMT10C, Asp314 forms a hydrogen bond with the NH2 group of A9 or is situated close to G9, suggesting it may serve as a general base to abstract a proton from the N1 position and enable the m^1A9 modification (B). In METTL6, Phe158 and Phe57 stabilized the targeted C32 base via π-interactions, while Arg60 and Arg213 formed hydrogen bonds with C32, creating a deprotonated state for nucleophilic attack (C). In NSUN6, Asp323 and Lys248 stabilize the targeted C72, while two active site residues (Cys373 and Cys326) act as nucleophiles to attack the C5 atom and create a covalent enzyme-nucleotide intermediate and a general base to remove the proton from C6 atom following methylation (D). In METTL1, Asp163, Arg109, Glu240, Asp199 and His26 are involved in G46 stabilization. Asp163 is thought to activate the N7 atom of G46 for nucleophilic attack (E). F and G, In archaeal Trm1-mediated m^2G and $m^2{}_2G$ modifications, Asp138 acts as a general base to abstract a proton from the N2 atom of G (or m^2G), enabling further methylation (F). The catalytic mechanisms of homologous tRNA MTases. In *Escherichia coli* (*E. coli*) TrmH, Arg41 stabilizes the target G nucleotide and deprotonates the 2′-OH group for Gm modification (G).

mitochondrial $tRNA^{Thr}$ and $tRNA^{Ser}$, potentially assisted by threonyl-tRNA synthetase (TARS2) or seryl-tRNA synthetase (SARS2) (Han et al., 2017; Huang et al., 2023b; Lentini et al., 2022). Particularly, the cytosolic $tRNA^{Ser}$ possesses a distinctively long variable loop compared to other tRNAs. Structural studies have revealed a unique m^3C methyltransferase-specific RNA-binding domain (m^3C-RBD) within METTL6, which plays a key role in recognizing the tRNA anticodon arm and facilitating selective binding with SerRS (Figure 3C). SerRS, in turn, promotes the specific recognition of $tRNA^{Ser}$ isoacceptors by

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interacting with the characteristic variable arm of the tRNA. Additionally, SerRS stabilizes the METTL6-tRNA complex, positioning C32 for base-flipping into the catalytic core of METTL6 for methylation. The structure revealed that aromatic residues Phe158 and F57 stack the C32 base via π-interactions (Figure 4C). The nucleophilic N3 atom of C32 is positioned near the sulfur atom of SAM, and Arg60 and Arg213 interact with C32 through hydrogen bonding, suggesting a deprotonated state for the nucleophilic attack (Throll et al., 2024). This coordinated interaction between METTL6 and SerRS not only ensures substrate specificity but also enhances METTL6's methylation efficiency, revealing a secondary "moonlighting" function for SerRS beyond its canonical role in aminoacylation. The structural flexibility of METTL6 m^3C-RBD and its selective interaction with SerRS underscores a distinctive recognition mechanism, providing a foundation for further studies on other members of the m^3C methyltransferase family (Cui et al., 2024).

NSUN6: docking the tRNA with a U-turn tail

The m^5C modifications are catalyzed by the NOL1/NOP2/Sun (NSUN) family and DNA methyltransferase 2 (DNMT2), both of which belong to class I MTases (Zhang et al., 2021b). DNMT2 was originally considered to be DNA MTase but proved to methylate C38 of tRNAs (Jeltsch et al., 2017). NSUN2 mediates m^5C modifications at positions 48, 49, and 50 in the variable loop and at position 34 in the anticodon loop, contributing to tRNA stability and protein synthesis (Van Haute et al., 2019). In mitochondria, NSUN3 is essential for m^5C34 modification (Nakano et al., 2016). Meanwhile, NSUN6 mediates the methylation of cytosine 72 near the 3′ end of tRNA, requiring the presence of the 3′-CCA motif for this modification (Haag et al., 2015).

Crystallographic studies have revealed that NSUN6 induces a U-turn conformational change at the 3′ end of the tRNA, breaking the base-paired region and allowing C72 to enter the catalytic pocket (Figure 3D). The Asp323 and Lys248 in NSUN6 stabilize the orientation of the target C72, while two conserved cysteine residues, Cys326 and Cys373, participate in the methyl transfer (Figure 4D). Cys373 acts as a nucleophile, attacking the C6 atom of C72 to form a covalent protein-RNA intermediate. The C5 atom of C72 is then activated to accept the methyl group from SAM, and Cys326 serves as a general base, abstracting a proton from C5 to complete the reaction (Liu et al., 2017). The PUA domain of NSUN6 precisely recognizes the 3′-CCA motif and interacts extensively with the D-arm of the tRNA. In addition to the CCA tail and D-arm, the discriminator base U73 and the target C72, which interact with two conserved catalytic cysteine residues, are key elements contributing to the specific recognition of the tRNA substrate. This study provides valuable insight into the specific substrate recognition and the well-characterized catalytic mechanism of tRNA MTases (Liu et al., 2017).

METTL1/WDR4: securing the tRNA for m^7G46 methylation

The m^7G46 modification of tRNA is catalyzed by METTL1, a class I MTase, in complex with its cofactor WDR4 (Lei et al., 2023). At this internal modification site in tRNAs, METTL1/WDR4 encounters a similar challenge in depositing the m^7G46 modification. The METTL1/WDR4 complex forms an active heterodimer, creating a positively charged surface that recognizes tRNA through complementary shape and charge interactions (Figure 3E) (Ruiz-Arroyo et al., 2023). This complex engages with the tRNA core region to secure the variable loop (containing target G46 nucleotide) by different elements, including αC helix (also named catalytic loop), α6 helix, the N terminal region of METTL1 and the C-terminal region of WDR4 (Zhang, 2024). The binding of the SAH cofactor triggers conformational changes in METTL1, repositioning the αC helix to trigger the methylation process. The N-terminal region of METTL1 is essential in forming the catalytic pocket and flipping the G46 base out of the tRNA core for modification. The proposed methylation mechanism follows an SN2-type reaction, with Asp163 and Arg109 in the catalytic pocket positioning G46 near the methyl group of SAM (Figure 4E). Additionally, Glu240 and His26 stabilize this conformation. Asp163 likely activates the N^7 atom of G46, while Glu240 and Asp199 facilitate deprotonation, enabling methyl transfer (Lei et al., 2023); (Ruiz-Arroyo et al., 2023). Although the 'RAGGU' motif has been identified in m^7G46-modified tRNAs, current structural studies have yet to fully elucidate how METTL1/WDR4 achieves specific substrate selectivity across different tRNAs.

Probing catalytic functions of uncharacterized tRNA MTases

Although detailed structural and mechanistic studies of other human tRNA MTases are limited, insights can be gained from homologous enzymes. For example, studies on TRMT1, which catalyzes m^2G and $m^2{}_2G$ modifications in archaea, suggest that an aspartate residue functions as a general base, abstracting a proton from the N^2 atom to facilitate methylation (Figure 4F) (Awai et al., 2011; Xiong et al., 2023).

Similarly, the 2′-O-methylatransferase TARBP1 possesses a SPOUT domain (class IV MTase) and catalyzes Gm18 modification (Boriack-Sjodin et al., 2018). Although the structure of its catalytic domain has been determined, the detailed catalytic mechanism remains unclear. Based on the mechanism proposed for *E. coli* TrmH, the active site arginine stabilizes the 5′-phosphodiester bond of the target nucleotide while deprotonating the 2′-OH, facilitating nucleophilic attack on the SAM methyl group (Figure 4G) (Persson, 1997). In addition to stand-alone methyltransferases that directly recognize tRNA, tRNA 2′-O-methylation (Nm) can also be guided by small nucleolar RNAs (snoRNAs) and catalyzed by fibrillarin (FBL) (Zhang et al., 2023b; Zhou et al., 2024).

Moreover, with the rapid advancements in structural prediction, such as AlphaFold3, we can now gain insights into the interactions between methyltransferases and their substrates (Abramson et al., 2024). We attempted to predict the structures of unresolved tRNA MTases bound to their tRNA substrates. While some predictions appear plausible, they still need to be validated. For example, in the predicted structure of the METTL2-DALRD3-tRNA, the overall architecture is generally reasonable but exhibits several potential limitations: certain regions could not be confidently predicted, and key residues essential for m^3C32 modification, as well as conserved motifs among homologous family members, do not exhibit similar interaction features. Notably, the anticodon region of the tRNA undergoes conformational changes upon interaction; however, instead of flipping into the conserved catalytic pocket, the target cytosine at position 32 extends in the opposite direction, which may indicate an intermediate state. By superposing the predicted structure

with the experimentally resolved METTL6 complex, we observe that they share similar catalytic residues, suggesting a conserved mechanism. These findings offer valuable insights for advancing our understanding of the functions and catalytic mechanisms of human tRNA MTases.

Substrate recognition in tRNA MTases: balancing flexibility and selectivity

Although many tRNA MTase structures have been determined, either alone or with their cofactors, the exact mechanisms by which they recognize and modify their tRNA substrates are not fully understood. Capturing these MTases in action with their substrate tRNA is particularly challenging due to the dynamic structural rearrangements of tRNA, which are critical to the catalytic process.

Most of the tRNA MTases modify a subgroup of tRNAs. Thus, they need to have a degree of flexibility to accommodate different tRNA molecules, typically relying on large electropositive surfaces to attract the tRNAs. However, tRNAs share similar negatively charged L-shape structures, tRNA MTases must also select their own ones from the crowded tRNA pool. To achieve this, they may need to employ a combination of methods, including initial recognition via general shape and charge properties, followed by more precise identification of specific sequence motifs and target sites, potentially aided by additional tRNA discrimination factors, etc. For example, the 'RAGGU' motif is essential for METTL1 to modify the G46; METTL2 and METTL8 coordinate the pre-modified t^6A37 to recognize the threonine tRNAs; NSUN6 utilizes the base U73 as a discriminator (Adams et al., 2018; Kleiber et al., 2022; Liu et al., 2017; Mao et al., 2021).

An intriguing phenomenon observed in several methyltransferase-cofactor-tRNA structures is the non-1:1:1 stoichiometries in their complexes. For example, the TRMT61A-TRMT6-tRNA complex adopts a 2:2:2 assembly that is critical for its catalytic activity, while the TRMT10C-SDR5C1-tRNA complex appears to have a 2:4:2 architecture (Finer-Moore et al., 2015; Meynier et al., 2024). Similarly, the METTL6-SerRS-tRNA complex has been found to adopt varying stoichiometries (1:2:1, 1:2:2, and 2:2:2), with comparable intermolecular contacts across these arrangements (Throll et al., 2024). These varying stoichiometries highlight the complexity of tRNA modification systems, underscoring the need for precise structural configurations to ensure substrate specificity, catalytic efficiency, and regulation.

tRNA MTase in developmental disorders and cancers

tRNAs were once considered universally expressed housekeeping molecules, but recent findings indicate that both tRNA expression and modifications exhibit tissue- and cell-type specificity (Ando et al., 2023; Dittmar et al., 2006; Hoffmann et al., 2018; Pinkard et al., 2020). Importantly, tRNAs are subject to extensive regulation within cells, and dysregulation of functional tRNAs can have profound effects, as reviewed in detail elsewhere (Orellana et al., 2022; Su et al., 2020; Yang et al., 2024). Particularly, aberrant post-transcriptional modifications of tRNAs play critical roles in tRNA dysregulation (Gao et al., 2022; Phizicky and Hopper, 2023; Suzuki, 2021). Recent studies have uncovered strong associations between tRNA methyltransferase and various human diseases. Here we summarize the identified patient cases, disease models, and the underlying pathological mechanisms, including m^1A, m^1G, m^3C, m^5C, m^7G, m^2G, and the Nm MTase, mainly in cancer, neurological and developmental disorders.

tRNA m^1A & m^1G methyltransferase

The m^1A and m^1G modifications sometimes share the same methyltransferase, thus, we discuss them together. TRMT61A, in complex with TRMT6, elevates m^1A58 methylation in a subset of tRNAs, activating the Hedgehog signaling pathway through PPARδ translation and cholesterol synthesis, leading to liver cancer stem cells (CSCs) and tumorigenesis (Wang et al., 2021). TRMT61A/TRMT6 is also implicated in bladder cancer (BC), likely through m^1A58 methylation on 3′ tRNA fragments, which disrupts seed pairing in mRNA silencing targets involved in the unfolded protein response (UPR) pathway (Su et al., 2022). Furthermore, recent studies have linked TRMT61A/TRMT6 to hematopoietic regeneration and hematopoietic stem cells (HSCs) aging, which is associated with myeloid malignancies (He et al., 2024; Zuo et al., 2024). Meanwhile, TRMT61B, the mitochondrial m^1A58 methyltransferase, is associated with breast and gastric cancers, and a recent study has expanded TRMT61B's involvement across a wide range of cancer types (Chujo and Suzuki, 2012; Couch et al., 2016; Li et al., 2021b; Martín et al., 2023). Further research is required to fully elucidate the mechanisms by which these MTases contribute to cancer pathogenesis.

In mitochondria, TRMT10C, in complex with SDR5C1, catalyzes the m^1R9 modification and serves as a platform for the assembly of pre-tRNA maturation enzymes, ensuring proper tRNA maturation (Meynier et al., 2024; Reinhard et al., 2017; Vilardo et al., 2012). Defects in TRMT10C may disrupt mitochondrial tRNA maturation, leading to a cascade of downstream consequences. Variants of TRMT10C, such as R181L and T272A, have been identified in patients with mitochondrial disorders, presenting at birth with symptoms including lactic acidosis, hypotonia, feeding difficulties, and deafness (Metodiev et al., 2016). Structural studies have revealed that these disease-associated mutations in TRMT10C are involved in tRNA interaction and are crucial for maintaining the fold of its catalytic domain (Meynier et al., 2024). Additionally, hypo-m^1A modification in tRNAs has been implicated in Alzheimer's disease (AD) pathogenesis (Figure 5). In an AD mouse model (5XFAD), tRNAs exhibit reduced m^1A methylation due to decreased expression of TRMT10C and the m^1A58 MTases TRMT61A (Shafik et al., 2022). Knockdown of these MTases in a Drosophila tau model exacerbates AD-related phenotypes (Shafik et al., 2022). Although the specific mechanisms by which these m^1A MTases contribute to AD pathogenesis remain unclear, mitochondrial dysfunction may be a contributing factor. Further patient case studies are needed to elucidate the underlying pathogenic and regulatory mechanisms.

TRMT10A and TRMT10B are responsible for depositing the m^1G9 and m^1A9 modification in the cytoplasm, respectively (Howell et al., 2019; Vilardo et al., 2020). Deficiencies in TRMT10A have been reported to cause microcephaly, diabetes, and intellectual disability (ID) (Figure 5) (Cosentino et al., 2018; Gillis et al., 2014; Igoillo-Esteve et al., 2013; Yew et al., 2016). A recent study demonstrated that the loss of TRMT10A reduces the

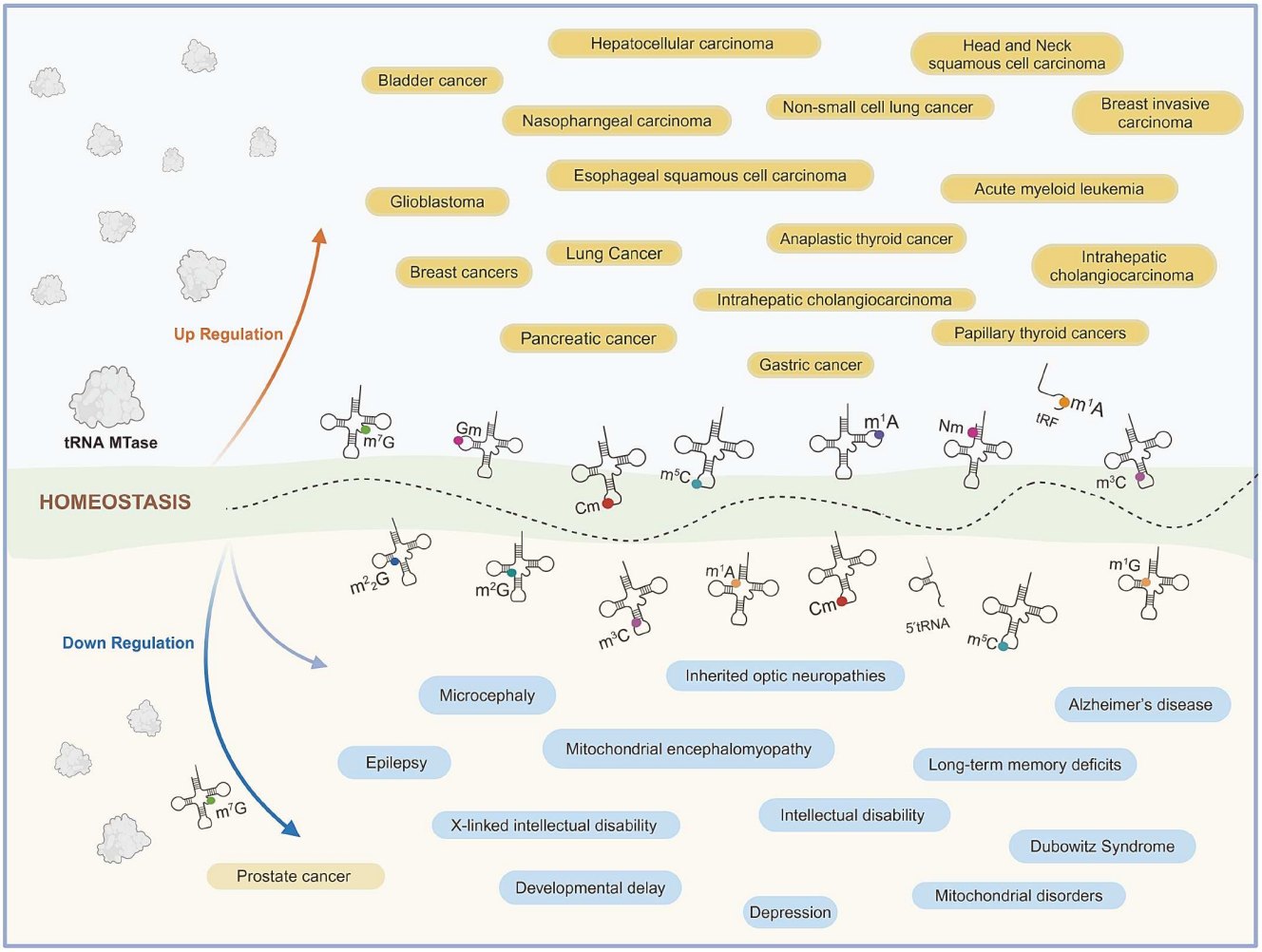


Figure 5. Dysregulation of tRNA MTases disrupts tRNA methylation homeostasis and drives disease pathogenesis. In a normal cellular environment, methylated tRNAs are dynamically maintained within a balanced homeostatic state. Dysregulation of tRNA MTases (gray) can disrupt the delicate equilibrium. Increased levels of some methylated tRNAs can promote tumorigenesis or contribute to cancer progression, while decreased methylation mainly leads to developmental and neurological disorders. The orange and blue rounded boxes in the figure highlight the names of the associated diseases.

levels of $tRNA^{iMet}$ and $tRNA^{Gln}$ across various tissues in mice, disrupting the translation of mRNAs involved in neuronal structure, leading to impairments in synaptic architecture, synaptic plasticity, and memory (Tresky et al., 2024). Unlike TRMT10A, TRMT10B displays a more selective substrate specificity, primarily targeting $tRNA^{Asp}$, and exhibits relatively lower catalytic activity *in vitro* (Howell et al., 2019). Despite the limited studies available, the conserved presence of TRMT10B across vertebrates suggests it holds a distinct biological function that remains to be fully elucidated.

tRNA m^3C methyltransferases

Similar to the m^1A MTase family, the m^3C MTases have expanded to four proteins in humans: METTL2A, METTL2B, METTL6, and METTL8, each responsible for modifying different tRNA substrates at position 32 (Mao et al., 2021; Xu et al., 2017). Impaired m^3C modification has been implicated in both physiological processes and disease. METTL2A and METTL2B are highly homologous, differing by only six amino acids, and both exhibit aberrant expression in various cancer types. Notably, METTL2A has been extensively studied as a potential oncogene in breast invasive carcinoma (BRCA) (Figure 5) (Wang et al., 2022). In addition to the catalytic methyltransferase, alterations in associated partner proteins that facilitate the normal catalytic process have also emerged as key targets in neurological pathogenesis. In humans, DALRD3 has been identified as forming a complex with m^3C MTase METTL2 and is required to catalyze m^3C formation in specific tRNAs ($tRNA^{Arg}CCU/UCU$) (Lentini et al., 2020). Patients with developmental delay and early-onset epileptic encephalopathy who possess a DALRD3 frameshift variant exhibit a nearly complete loss of m^3C modification in arginine tRNAs, implicating DALRD3 in neurological disorders (Lentini et al., 2020). Recently, the same research group identified a pathogenic variant in DALRD3, where an arginine residue is substituted by cysteine (R517C) (Zhang et al., 2024a). This variant impairs m^3C modification in arginine tRNAs by reducing the association of DALRD3 with METTL2 and its tRNA substrate (Zhang et al., 2024a). These findings emphasize the crucial role of DALRD3-dependent tRNA

modification in proper brain development and neurological function. More works, like experimental structure determination of DALRD3 or in complex with METTL2-tRNA, are needed to elucidate the catalytic and pathogenic mechanism behind DALRD3-dependent tRNA modifications.

In hepatocellular carcinoma (HCC), METTL6 has been identified as a key regulator of pluripotency and tumor cell growth (Ignatova et al., 2020). Studies have shown that METTL6 post-transcriptionally modulates cell adhesion proteins, such as ITGA1, SPON1, and CLDN14, to influence cancer cell behavior (Bolatkan et al., 2021). Recent research has also revealed METTL2/6-dependent methylomes and a serine codon-biased mRNA translation that affects genes involved in the cell cycle and DNA repair (Cui et al., 2024). Notably, METTL6 specifically catalyzes the m^3C32 modification on the tRNA-serine family, a role distinct from METTL2A/2B. A decrease in m^3C32 on $tRNA^{Ser}$ was only observed with the combined deletion of METTL2A/2B and METTL6 (Cui et al., 2024). Although the complexity of the m^3C epitranscriptome in human cells remains to be explored, METTL6 has emerged as a promising therapeutic target in HCC.

METTL8 catalyzes the m^3C32 modification on mitochondrial mt-$tRNA^{Thr/Ser}$, a modification essential for proper mitochondrial function (Huang et al., 2022; Huang et al., 2023b; Kleiber et al., 2022). Elevated METTL8 expression has been linked to poor patient survival in pancreatic cancer (PC), while its deficiency results in impaired maintenance of embryonic cortical neural stem cells (Schöller et al., 2021; Zhang et al., 2023a). These effects are attributed to disruptions in mitochondrial protein translation, mediated by METTL8's role in modifying specific codon tRNAs. A recent study also links METTL8 to glioblastoma (GBM) stemness and tumorigenicity (Figure 5) (Lee et al., 2024). However, the understanding of m^3C methylation remains limited, and further research is needed to elucidate the underlying mechanisms and identify the associated regulatory proteins.

tRNA m^5C methyltransferases

NSUN2 is a multifunctional enzyme that catalyzes the deposition of m^5C to tRNAs, mRNAs, and non-coding RNAs, influencing various biological processes and contributing to tumorigenesis (reviewed elsewhere (Chellamuthu and Gray, 2020; Li and Huang, 2024)). Here we summarize exclusively on NSUN2's role in tRNA modification and its associated pathological implications. Loss-of-function mutations in NSUN2 have been linked to autosomal recessive intellectual disability, microcephaly, and Dubowitz Syndrome (DS) (Abbasi-Moheb et al., 2012; Flores et al., 2017; Khan et al., 2012; Komara et al., 2015; Martinez et al., 2012). NSUN2-deficient models demonstrated that loss of NSUN2 increases hypomodified tRNAs, resulting in their cleavage and accumulation of 5′ tRNA fragments during brain development in mice (Blanco et al., 2014; Flores et al., 2017). Additionally, NSUN2 has been shown to influence neuronal synapse function and behaviors related to depression (Blaze et al., 2021). More recently, decreased NSUN2 expression has been implicated in promoting neurodegeneration by regulating tau phosphorylation, suggesting NSUN2 as a potential therapeutic target for AD (Kim et al., 2023). Conversely, NSUN2 upregulation promotes codon-dependent oncogenic translation in anaplastic thyroid cancer (ATC). In mitochondria, NSUN3 is responsible for the methylation of cytosine 34 (C34) of mt-$tRNA^{Met}$, a process that is followed by ALKBH1-mediated conversion to f^5C, both of which are critical for maintaining normal mitochondrial function (Haag et al., 2016; Nakano et al., 2016; van Haute et al., 2016). The deficiency of NSUN3 can cause early-onset mitochondrial encephalomyopathy, seizures, and inherited optic neuropathies (IONs) (de Muijnck et al., 2024; Paramasivam et al., 2020). Meanwhile, the study also showed that upregulation of NSUN3 promotes tumor progression of head and neck squamous cell carcinoma (HNSCC) (Figure 5) (Jin et al., 2024). By mediating m^5C modifications in mitochondrial tRNAs, NSUN3 enhances the translation of mitochondrial mRNAs, supporting cancer cell metastasis and powering cancer cells to invade and spread (Delaunay et al., 2022). This highlights NSUN3 as a potential therapeutic target in cancer treatment.

Beyond the NSUN family, DNMT2 is upregulated in different cancer types, and disease-related mutations have been identified (reviewed elsewhere (Gu et al., 2023; Jeltsch et al., 2017)). DNMT2 mediates the m^5C38 modification on tRNAs, which has been linked to queuosine and m^2G modifications, highlighting the complexity of its biological functions. The next section will discuss inhibitors targeting DNMT2.

tRNA m^7G methyltransferases

METTL1, along with its cofactor WDR4, mediates the m^7G46 modification in tRNAs and plays a pivotal role in various physiological processes and diseases (Luo et al., 2022). METTL1-WDR4-mediated m^7G modification increases the abundance of certain tRNAs, enhancing the translation of oncogenes through codon preference, thereby promoting tumor progression. Notably, m^7G-modified $tRNA^{Arg}$ (TCT) has been identified as significantly increasing the translation of cell cycle regulators enriched in AGA codons, thus promoting tumorigenesis (Orellana et al., 2021). In the study of intrahepatic cholangiocarcinoma (ICC), METTL1-WDR4 promotes cancer progression in a $tRNA^{Lys}$ (CTT) and $tRNA^{Lys}$ (TTT) codon-dependent manner (Dai et al., 2021). Moreover, METTL1 knockdown has been shown to reduce resistance to lenvatinib in HCC, suggesting that METTL1 could serve as a potential biomarker for lenvatinib sensitivity and offer new therapeutic strategies through drug combination (Huang et al., 2023a). Recent studies also indicate that METTL1-mediated m^7G modification plays a complex role in prostate cancer (PCa), where the loss of this modification leads to tRNA fragmentation and inhibits the initiation of translation of related genes, thereby restricting PCa growth (Figure 5) (García-Vílchez et al., 2023).

As the non-catalytic subunit of the m^7G methyltransferase, WDR4 promotes the methyltransferase activity of METTL1 by acting as a scaffold that facilitates tRNA binding and enhances the recognition of the SAM (Jin et al., 2023; Lei et al., 2023; Li et al., 2023; Ruiz-Arroyo et al., 2023). Mutations in the WDR4 gene have been associated with human developmental disorders, including intellectual disability, growth retardation, and microcephaly (Braun et al., 2018; Shaheen et al., 2015; Trimouille et al., 2018). Patient-derived mutations in WDR4, such as arginine to leucine or glutamine substitutions (R170L/Q), compromise its structural rigidity as a scaffold, thereby affecting m^7G deposition by METTL1 (Li et al., 2023). In patients homozygous for the WDR4-R170L allele, the m^7G modification level of $tRNA^{Phe}$ is significantly reduced, highlighting the critical role of m^7G-

mediated gene regulation in neurological development (Shaheen et al., 2015). Further research on WDR4-deficient models is necessary to elucidate the molecular mechanisms underlying these pathologies. These studies provide critical insights into the biological roles of METTL1-WDR4 and their contributions to disease development, highlighting targeting tRNA m^7G modification offers promising therapeutic strategies for cancers and other diseases, holding significant value for drug development.

tRNA m^2G & $m^2{}_2G$ methyltransferases

TRMT1 catalyzes the m^2G26 and $m^2{}_2G26$ modifications in over half of human tRNAs, and its deficiency has been associated with developmental delay, ID, and epilepsy (EP) (Figure 5) (Blaesius et al., 2018; Davarniya et al., 2015; Xiong et al., 2023). Studies have shown that TRMT1 variants in ID patients, including frameshift and missense mutations, lead to a loss of catalytic activity due to defects in RNA binding or disruption of catalytic domain folding (Dewe et al., 2017; Zhang et al., 2020). These alterations in TRMT1 activity impair protein synthesis; however, the precise mechanisms by which this disruption leads to downstream effects contributing to the pathogenesis of neurological disorders remain unclear. Although TRMT1L is less well understood, recent findings indicate that it catalyzes the $m^2{}_2G27$ modification specifically on $tRNA^{Tyr}$ and is essential for efficient mRNA translation and cell survival under oxidative stress, suggesting it may have similar pathogenic roles to TRMT1 (Hwang et al., 2024; Zhang et al., 2024b).

TRMT112 functions as a cofactor hub, coordinating with THUMPD3 or TRMT11 to facilitate m^2G deposition at various sites (Brūmele et al., 2021; Yang et al., 2021). However, the precise mechanisms by which TRMT112 stimulates this activity remain unclear. Increased TRMT112 expression has been observed in several tumor types, indicating its potential as a prognostic marker (Xu et al., 2022). Knockout studies of THUMPD3 reveal impaired global protein synthesis and reduced cellular growth, potentially linked to sperm tRNA-derived fragments (Yang et al., 2021). Despite these findings, the biological and pathogenic roles of these m^2G MTases and their cofactors remain largely unexplored and warrant further investigation.

tRNA 2′-O-methyltransferase

The 2′-O-methyltransferases, depositing a methyl group to the 2′ hydroxyl of the ribose moiety, are involved in various diseases (Dimitrova et al., 2019). FTSJ1, implicated in X-linked intellectual disability (XLID), is responsible for Nm modifications in a subset of cytosolic tRNAs (Freude et al., 2004; Guy et al., 2015). The absence of Cm32 modification on $tRNA^{Phe}$ (UUU) leads to a selective reduction of tRNA levels in the brain, affecting the translation of mRNAs linked to brain dysfunction, and recent studies suggest that FTSJ1 deficiency alters neuronal morphology and may contribute to long-term memory deficits (Brazane et al., 2023; Li et al., 2020; Nagayoshi et al., 2021). FTSJ1 forms complexes with WDR6 or THADA to modify specific tRNAs, with brain-specific changes in tRNA levels, particularly $tRNA^{Phe}$, potentially due to high RNase activity in the brain, which renders hypomodified tRNAs more susceptible to degradation (Li et al., 2020; Nagayoshi et al., 2021). Moreover, FTSJ1 has been shown to suppress tumor growth in non-small cell lung cancer (NSCLC) by downregulating DRAM1, while in triple-negative breast cancer (TNBC), it acts as a tumor promoter and is involved in immune evasion (Figure 5) (He et al., 2020; Sun et al., 2024).

The 2′-O-methyltransferase TARBP1 has been reported to have the highest mutation rate (1.86%) in a pan-cancer analysis (Manning et al., 2020). A recent study has identified TARBP1 as a key regulator of glutamine metabolism and tumorigenesis, with its upregulation linked to poor prognosis in HCC (Figure 5) (Shi et al., 2024). Additionally, they show that TARBP1 facilitates Gm18 methylation of tRNAs, particularly $tRNA^{Gln}$ (CTG), which enhances tRNA stability and promotes the synthesis of the glutamine transporter ASCT2 (Shi et al., 2024). The loss of TARBP1 function impairs glutamine uptake, a key process as cancer cells heavily relies on both glucose and glutamine for growth. This highlights TARBP1 as a promising therapeutic target in cancer treatment. However, the mechanisms driving TARBP1's substrate specificity for $tRNA^{Gln}$ (CTG) and the role of TARBP1 mutations in disease remain poorly understood and warrant further investigation.

Nm4 is a conserved tRNA modification in eukaryotes, and the methyltransferase responsible for this modification in humans, TRMT13 (previously known as CCDC76), has only recently been characterized (Li et al., 2022; Wilkinson et al., 2007). Current evidence suggests that TRMT13 is associated with several cancers, including breast and papillary thyroid cancers (Li et al., 2022; Liu et al., 2024). However, the role of TRMT13-mediated Nm4 modification, as well as its potential non-catalytic functions in disease, remains to be fully elucidated and requires further investigation.

tRNA methylation homeostasis and disease: When balance is broken

In a healthy and normal physiological environment, tRNA methylation modifications are typically maintained in a state of homeostasis. Disruptions to this balance, such as hypomethylation or hypermethylation mediated by dysregulated tRNA MTases, can lead to abnormal translation processes or result in the accumulation of tRNA fragments, which in turn contribute to the pathogenesis of various diseases (Figure 5).

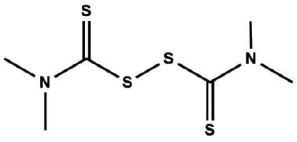
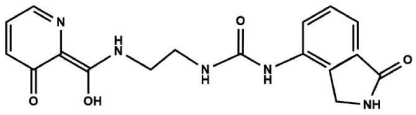
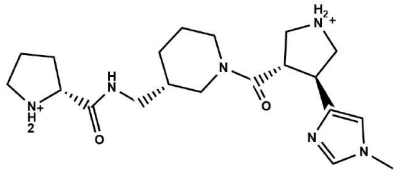
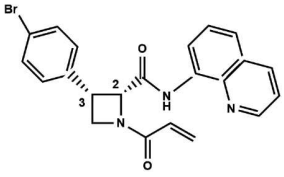
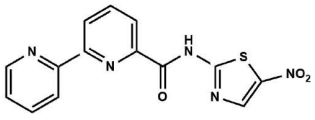
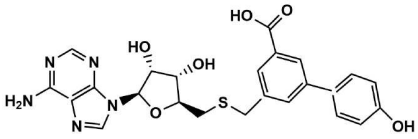
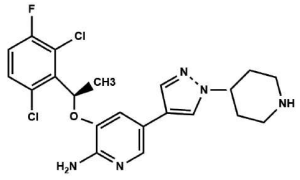
Most of the pathogenic mechanisms discussed in this review are dependent on the catalytic activity of tRNA MTases. Loss-of-function mutations or gene amplifications in tRNA MTases can cause downregulation or upregulation of tRNA methylation levels, respectively. Notably, in most cases, upregulated tRNA methylation is associated with tumorigenesis, positioning these tRNA MTases as potential oncogenes. In contrast, downregulation is often linked to developmental and neurological disorders. However, some tRNA MTases exhibit context-dependent dual roles; for instance, METTL1 acts as an oncogene in many cancers but displays tumor-suppressive functions in prostate cancer (García-Vílchez et al., 2023). Additionally, certain tRNA MTase-mediated pathologies demonstrate codon-dependent mechanisms or show tissue-specific features such as brain disorders. These enzymes interact dynamically with various proteins and may be implicated in multiple cellular pathways, requiring precise regulation to maintain homeostasis. The non-classical roles of tRNA MTases, which are not covered in detail here, suggest additional layers of complexity in understanding the relationship between tRNA MTases and disease. Despite some unresolved questions, current research provides critical insights

into the molecular mechanisms underlying tRNA modification-related disorders and underscores the potential of targeting tRNA MTases for therapeutic intervention.

Progress in tRNA methyltransferase drug hunting

Compared to drug development efforts targeting DNA methylation and histone modification-related proteins, RNA modifications, particularly tRNA methylation, remain less explored and targeted. The discovery of small-molecule inhibitors for METTL3 (m^6A MTase), which showed significant anti-leukemic effects in preclinical models, greatly highlights the potential of pharmacologically targeting RNA MTases as a novel therapeutic strategy (Li and Gregory, 2021; Moroz-Omori et al., 2021; Yankova et al., 2021). With the growing recognition of the critical role of tRNA methylation in various physiological processes and diseases, interest in developing inhibitors for tRNA MTases has increased. However, reports on tRNA MTase inhibitors are still limited, with most findings emerging within the last four years. Here, we summarize recent advancements in this area (Table 2).

Table 2. Potential small molecule compounds targeting tRNA Mtase

tRNA MTase target	Potential compound	Inhibitor qualities	Mechanism of inhibition	Biological validation
TRMT6/ TRMT61A	Thiram 	Efficient inhibitor against TRMT6/TRMT61A's activity for m^1A modification	May disrupt TRMT6-TRMT61A interaction	Phenotypic drug discovery (PDD); significant reduction in tumor growth in liver cancer xenograft models; needs further *in vivo* optimization and detailed mechanistic exploration
DNMT2	Compound 1.4 	K_D=37 μmol L^{-1}; off-target on NSUN6	Targets SAM binding site	Virtual screening; validated in binding affinity; no inhibition potency data; needs significantly improved selectivity; considered as initial hits for further optimization
NSUN6	Compound 2.4 	K_D=16.4 μmol L^{-1}; high selectivity for NSUN6	Targets SAM binding site	Virtual screening; validated in binding affinity; no inhibition potency data; considered as initial hits for further optimization
NSUN2	MY-1B 	IC_{50}=1.3 μmol L^{-1}; high selectivity for NSUN2	Covalent binding to the conserved catalytic Cys residue	Activity-based protein profiling (ABPP); reduced m^5C levels in cancer cells; validated in liver cancer models; requires potency improvement and deeper mechanistic investigation
NSUN3	Compound B19 	IC_{50}=0.19 μmol L^{-1} in CRC models, highly selective for NSUN3	Detailed mechanisms are not clear	Phenotypic drug discovery (PDD); reduced tumor growth in colorectal cancer xenografts; and requires detailed mechanistic exploration and improved potency
METTL1	Compound 6 	Multiple compounds IC_{50}=40–300 μmol L^{-1} weak/moderate specificity	Targets SAM binding site	Virtual screening; validated in inhibitory potential; requires optimization for higher potency and selectivity
METTL1	(S)-crizotinib 	K_D=138 μmol L^{-1}; IC 50=158 μmol L^{-1}; high selectivity for METTL1	Targets SAM binding site	Fluorescence-based screening; validated in binding affinity and inhibition potency via multiple methods; requires optimization for higher potency

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In 2021, (Wang et al., 2021) investigated the role of TRMT61A/TRMT6 in liver cancer progression and identified Thiram as a potent inhibitor of the TRMT61A/TRMT6 complex through the screening of an FDA-approved drug library (comprising 1600 molecules). Thiram treatment was shown to significantly reduce m^1A levels and inhibit liver tumor growth in mouse models. However, the exact mechanism that Thiram uses to impair TRMT61A/TRMT6 catalytic activity has not been thoroughly studied.

In the case of m^5C MTases, azacytidine, a drug originally developed for DNA MTases, and several SAM homologous ligands have been shown to inhibit DNMT2 activity (Schaefer et al., 2009; Schwickert et al., 2022). In 2023, Zimmermann et al. (2023) discovered several small molecules capable of inhibiting DNMT2 and NSUN6 through high-throughput virtual screening. Among these, Compound 1.4 exhibited the highest binding affinity to DNMT2 but with moderate off-target binding to NSUN6, while Compound 2.4 showed strong specificity for NSUN6, providing promising starting points for further optimization. In the same year, using cysteine-directed activity-based protein profiling (ABPP), Tao et al. (2023) identified azetidine acrylamides as selective covalent inhibitors of NSUN2, particularly MY-1B, which targets the catalytic cysteine (C271) with high specificity and minimal cross-reactivity with other NSUN family members. In 2024, Tang et al. (2024) discovered compound B19 through a phenotypic drug discovery (PDD) strategy, a potent NSUN3 inhibitor with strong anti-tumor activity in colorectal cancer, though the exact mechanism of NSUN3 inhibition remains to be elucidated.

Recently, progress has been made in the development of METTL1 inhibitors through two key studies. Nai et al. (2024) identified four inhibitors targeting the SAM binding site using docking and enzymatic assays. Among these, Compound 6 emerged as the most potent, with an IC50 of 42 μmol L^{-1}, while the other three exhibited distinct substitution patterns. These initial small molecules hold promise for further optimization. Later, Meidner et al. (2024) developed an innovative fluorescence-based screening approach and identified three potent inhibitors, with (S)-crizotinib showing potential as a selective METTL1 inhibitor. However, additional optimization and biological validation are required for these inhibitors.

Given the role of tRNA methyltransferase dysregulation in various human diseases, inhibiting their catalytic activity presents an attractive therapeutic opportunity. The primary approach involves targeting the catalytic pocket, often using structure-based high-throughput virtual screening combined with functional assays to monitor MTase activity. Since RNA MTases rely on SAM as a cofactor, designing inhibitors that competitively bind to the SAM-binding pocket is a particularly effective strategy. However, because tRNA MTases share the SAM-binding site with other RNA, DNA, and protein methyltransferases, achieving selectivity remains challenging. As a result, competitive inhibitors targeting the SAM-binding pocket often display off-target effects due to the structural conservation of this site across the methyltransferase family. While some potential small molecule compounds discussed in the manuscript have been predicted to bind to the catalytic pocket of target MTases, their experimental structural characterization remains absent. Furthermore, certain homologs of the native cofactor SAM exhibit poor physicochemical properties and low drug-likeness, requiring optimization to enhance their potency, selectivity, and pharmacokinetics. Phenotype- or activity-based approaches have identified compounds with potent anti-disease effects, although their exact molecular targets and mechanisms often require further elucidation. With continued development, these compounds have the potential to evolve into effective inhibitors of RNA MTases, offering new therapeutic avenues for diseases associated with RNA methylation dysregulation.

Conclusion

The landscape of tRNA methylation is intricate, with tRNA MTases playing a central role in regulating tRNA stability and function. Given the complexity and diversity of the tRNA family, tRNA MTases demonstrate remarkable substrate selectivity, targeting distinct sites on specific tRNAs. Disruption of this finely tuned process leads to aberrant methylation patterns, driving various diseases. While tRNA MTases present promising therapeutic targets for methylation-related disorders, several critical challenges persist: How do tRNA MTases achieve substrate specificity across different tRNA species and cellular contexts? What is the broader interaction network between tRNA MTases and other RNA modifications within the epitranscriptomic landscape? How do their non-catalytic roles influence cellular processes? For a single tRNA MTase linked to multiple diseases, does a common pathogenic pathway exist or do distinct mechanisms underlie each condition?

Moreover, a key challenge is developing selective inhibitors to minimize off-target effects, particularly when targeting the conserved SAM-binding pocket. To optimize both affinity and specificity, incorporating stereochemical complexity, as seen in natural products, into the design of inhibitors targeting conserved pockets may present a rational strategy. Identifying allosteric sites or unique tRNA-interacting residues distinct from conserved regions further enhances the potential for selective inhibition. As AI-powered tools advance, the rational design of inhibitors becomes more efficient, though it remains an iterative process requiring continuous refinement through both computational and experimental approaches. Progress continues in addressing these challenges, making tRNA MTases viable targets for novel therapeutic approaches.

Compliance and ethics

The authors declare that they have no conflict of interest.

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